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Isolation of Endophytic *Streptomyces* Strains from Surface-Sterilized Roots

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When the roots of 28 plant species were surface sterilized and incubated on agar medium, endophytic actinomycetes in the root cortex were observed by direct microscopic observation and pure culture techniques.

Actinomycetes represent a large part of the rhizosphere microbial flora, but root-microorganism interactions have been extensively studied only for the nitrogen-fixing *Frankia* species (1) and the phytopathogenic *Streptomyces scabies* (4). Few studies have been done on the presence of other genera in plant roots (6, 17). Several reports refer to actinomycete activity in plant protection against pathogens and to the influence of metabolic products of actinomycetes on plant growth and physiology (5, 7, 14, 15, 19).

As isolation material, we used samples of roots belonging to 28 species, all collected from plants that exhibited healthy vegetative growth. Samples were obtained during the whole year in different geographic and climatic environments located in northwestern Italy.

We did not follow a prearranged strategy in the choice of botanical species; the strategy was based more on actual interest or on a peculiarity of the plant. Ectomycorrhizal roots of *Betula pendula* and a *Quercus* sp. were included because of the peculiar relationship they have with fungi. A *Euphorbia* sp. was included because its roots produce a dense caustic secretion. Because of the explorative character of this research, we tried to test the greatest number of different species.

The starch-casein medium proposed by Küster and Williams (8) and 2.5% water agar were used as isolation media. To both media, we added 50 ppm of nystatin and 50 ppm of cycloheximide to suppress fungal growth (18).

Roots (1 to 5 mm in diameter), washed to remove soil particles, were surface sterilized by exposing them to propylene oxide vapors for 1 h. Then, aseptically cut pieces (about 1 cm) were incubated on the media described above for up to 21 days at 25°C. For each root sample, the greatest number of colonies showing different morphological characteristics was isolated.

Cultural and morphological characteristics (presence of aerial mycelium, spore mass color [16], distinctive reverse colony color, diffusible pigment, and sporophore and spore chain morphology) were recorded after 14 days of incubation on CAY (Czapek agar [Difco] plus 0.2% yeast extract) and T3 (International Streptomyces Project Medium 3; Difco).

Antimicrobial activity against *Escherichia coli* (ATCC 25922), *Micrococcus luteus* (ATCC 9341), and *Fusarium oxysporum* f. sp. *cyclaminis* (IPV FW-286) was tested in PGC broth (10 g of Proflo [Trader Protein Division] per liter, 15 g of glycerol per liter, 3 g of calcium carbonate per liter) after 10 days of incubation on a rotary shaker at 24°C.

For scanning electron microscopy, roots were prepared by a technique previously described (10).

After 4 to 7 days of incubation, the surfaces of the root pieces showed hyphal growth that had formed small colonies. These colonies then propagated to the agar surface. Fungal growth was almost completely inhibited by antibiotics, while bacterial contamination was lower on water agar than on starch-casein medium. On both media, actinomycete colonies were clearly detectable.

A raw quantitative estimation of the presence of actinomycetes, mostly *Streptomyces* species, on the roots of 13 of the 28 plant species examined is reported in Table 1. These figures clearly indicate that large actinomycete populations are present on all the tested plant roots. Nearly 100% of the fragments incubated on water agar were colonized, whereas sometimes starch-casein medium gave lower figures (*Festuca*, *Rubus*, and *Chelidonium* species); on both media, the number of colonies per fragment was highly variable.

Scanning electron microscopy studies done on cryofractured roots revealed streptomycete hyphal growth inside the cortical cells; these structures were surrounded and often partially coated by a mucilaginous layer (Fig. 1).

We isolated 499 actinomycetes, most of them (482) being *Streptomyces* strains; other strains belonged to *Streptoverticillium* ($n = 2$), *Nocardia* ($n = 4$), *Micromonospora* ($n = 1$), and *Streptosporangium* ($n = 1$) genera. Nine isolates never bore reproductive structures and could not be identified. The

TABLE 1. Number of actinomycete colonies per root fragment

Plant	Water agar				Starch-casein medium			
	n[a]	%[b]	Avg[c]	SD	*n*[a]	%[b]	Avg[c]	SD
Allium porrum	30	100	7.00	3.44				
Amaryllis belladonna	30	97	7.07	5.15	30	100	13.13	9.66
Betula pendula	30	100	11.57	6.80	20	100	19.75	14.67
Brassica oleracea	30	100	4.80	4.00	30	93	3.73	3.11
Calluna vulgaris	30	63	1.33	1.42	30	83	1.70	1.39
Chelidonium majus	20	85	5.70	6.30	20	15	0.60	1.50
Cichorium intybus	30	100	4.37	2.40				
Euphorbia sp.	20	100	14.50	10.87	20	80	9.25	11.37
Festuca rubra	30	100	5.37	3.51	30	40	1.13	1.83
Fragaria vesca	30	100	4.20	2.14				
Lactuca scariola	30	93	1.50	1.48	30	83	2.00	3.05
Quercus sp.	30	100	4.47	2.74	30	93	7.90	7.12
Rubus idaeus	30	100	6.63	3.17	30	23	1.77	4.63

[a] *n*, number of fragments.
[b] Percentage of colonized fragments.
[c] Average number of actinomycete colonies per fragment.

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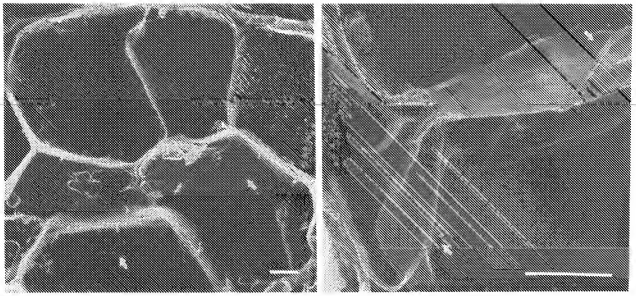

FIG. 1. Colonization of endophytic streptomycetes inside the cortical tissue of tomato roots. Arrows indicate the hyphae; bar, 5 μm.

number of *Streptomyces* strains isolated ranged from 3 for *Chelidonium majus* to 68 for *Glycine max* (Table 2).

On the basis of recorded characteristics, the *Streptomyces* strains were divided into 72 groups of identical microorganisms and 104 individual strains. Ten groups were composed of more than nine strains and were present in more than five different plants (Tables 2 and 3). The most common pattern of endophytic streptomycete seems to be represented by group A, isolated 45 times from 18 plant species of the 28 examined. Within groups F and J (Table 3) are assembled the most widespread antifungal activity-producing strains (23 isolates from 15 different plant species). This result needs

TABLE 2. Widespread *Streptomyces* isolates: distribution among the tested plants

Plant	IS[a]	DI[b]	Group									
			A	B	C	D	E	F	G	H	I	J
Allium porrum	12	11	+	+		+						
Amaryllis belladonna	24	16		+		+				+		
Betula pendula	26	16	+	+				+			+	
Brassica oleracea	7	7								+		
Calathea sp.	6	3		+								
Calluna vulgaris	21	19	+	+	+				+		+	
Camellia japonica	13	12			+							
Carex sp.	18	13	+			+	+				+	
Chelidonium majus	3	3	+									
Chrysanthemum indicum	8	8	+				+					
Cichorium intybus	8	7		+				+				
Cyclamen persicum	18	15	+	+			+	+	+	+		+
Euphorbia sp.	24	19		+		+		+			+	
Festuca rubra	27	19	+	+	+				+		+	
Fragaria vesca	26	15	+	+	+	+	+	+			+	
Glycine max	68	29	+	+		+	+	+	+			+
Hordeum vulgare	5	5	+			+						
Hyacinthus orientalis	6	6					+	+		+		
Lactuca scariola	11	6	+		+		+				+	
Medicago sativa	27	19	+	+	+					+		
Phragmites communis	12	11	+			+		+		+	+	
Quercus sp.	29	20	+	+		+	+				+	
Rubus idaeus	15	13	+		+		+	+				+
Saintpaulia kewensis	7	6	+		+							
Secale cereale	15	14		+	+					+		
Triticum aestivum	9	7			+	+	+					
Triticum durum	9	9										+
Vaccinium myrtillus	28	21	+			+	+	+	+		+	+

[a] IS, number of *Streptomyces* strains isolated per plant.
[b] DI, number of different strains within the isolates from the same plant.

TABLE 3. Widespread *Streptomyces* isolates: composition and characteristics of main groups

Group	No. of isolates	No. of plants in which isolate was present	Grey spore mass on T3	Red spore mass on T3	Yellow spore mass on T3	Yellow diffusible pigment on T3	*Rectiflexibiles* spore chains	*Retinaculiaperti* spore chains	Activity against:	
									M. luteus	*F. oxysporum*
A	45	18	+	–	–	–	+	–	–	–
B	29	14	–	+	–	–	+	–	–	–
C	20	10	–	+	–	–	–	+	–	–
D	19	11	+	–	–	–	+	–	+	–
E	16	11	–	–	+	–	+	–	–	–
F	14	10	+	–	–	–	+	–	–	+
G	12	5	+	–	–	–	–	+	–	–
H	12	7	–	–	+	–	+	–	+	–
I	11	10	+	–	–	+	+	–	–	–
J	9	5	–	–	+	–	+	–	–	+

more study, but it suggests an important role of these endophytic microorganisms in natural plant protection.

In columns 1 and 2 of Table 2 are reported, respectively, the number of *Streptomyces* strains isolated from each plant and the number of different strains within the isolates. The number of duplicate strains increased with the number of colonies isolated.

Incubation of surface-sterilized plant parts in a moist chamber and plating of tissues on agar media are techniques usually employed in plant pathology, and not often used in microbial ecology. They may be extremely useful in isolation of microorganisms from uncommon habitats. Using these techniques, we were able to confirm the presence of endophytic streptomycetes in root systems (11–13). In the present study, we demonstrated endophytic streptomyces in all the plant species examined by direct scanning electron microscopy and by isolation on agar plates. Colonization is restricted to the cortical layer, as described in the literature for endotrophic mycorrhizae and actinorrhizae (2, 3). The large number of *Streptomyces* strains isolated from healthy plants and the direct scanning electron microscopy investigations on internal tissues show that there is a close relationship between these microorganisms and roots, in which actinomycete hyphal growth could have a favorable effect.

The presence of streptomycetes inside the root tissues has an important role with regard to plant development and health. Their biological activities can interact with plant growth either by nutrient assumption or by the in situ production of secondary metabolites which stimulate or depress vegetative development. These microorganisms may also protect against soil-borne pathogens (9). Good results in biological control of plant diseases have been achieved with *Streptomyces* strains. The defensive effect could be achieved by root actinomycetes both by acting as competitors and by producing antibiotics and antifungal substances.

Further investigations are therefore necessary to understand the types of relationships between streptomycetes and plant tissues and the actual dynamics of this phenomenon.

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